OXIDATIVE STRESS AND ITS CORRELATION WITH GLYCATED HEMOGLOBIN IN PATIENTS OF TYPE2 DIABETES MELLITUS

A

Synopsis

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled, oxidative stress and its correlation with glycated hemoglobin in patients of type2 diabetes mellitus, in partial fulfilment of the requirements for the award of the Degree of Doctorate of Philosophy and submitted in the Department of Biochemistry of the Desh Bhagat University, Mandi Gobindgarh, Punjab is an authentic record of my own work carried under the supervision of **Dr. Manpreet kaur verma**, Assistant Professor Department of Chemistry, Desh Bhagat University, Mandi Gobindgarh and **Dr. Sukhraj kaur** Assistant Professor Department of Biochemistry, Govt. Medical college Amritsar.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other university.

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INTRODUCTION

Absolute or relative deficiency of insulin produces diabetes mellitus [1]. A large number, approx 382 million people worldwide or 8.3% of adults are estimated to be suffering from Diabetes. About 80% of them are living in the low and middle income countries. Continuing trends may result in one adult in ten having diabetics. This equates to approximately three new cases every 10 seconds or almost 10 million per year. The largest increases will take place in theregions where developing economies are predominant [2]. Hyperglycaemia generates reactive oxygen species (ROS), which in turn cause damage to the cells in many ways. Damage to the cells ultimately results in secondary complications in DM. [3] Oxidative stress plays a pivotal role in cellular injury from hyperglycaemia. High glucose level can stimulate free radical production. Free radical production can accelerate free radical generation and ROS. This enhanced ROS generation may not be counteracted by the weakened/weak immune system of the body and results in a condition called oxidative stress [4].

Classification:

Type 1 Diabetes Mellitus, Type 2 Diabetes Mellitus, Gestational Diabetes Mellitus and Other Specific Types.

Type 1 Diabetes Mellitus (T2DM): Type 1 diabetes occurs when the body's own immune system destroys the insulin- producing beta cells of the pancreas. Type1 diabetes affects children and adults of young age [5].

Type 2 Diabetes Mellitus (T2DM): Type 2 diabetes mellitus is the most common type of diabetes characterized by insulin resistance, which may be combined with relatively reduced insulin secretion. Defective responsiveness of body tissues to insulin is believed to involve the insulin receptor. Age cannot be used to classify T1DM & T2DM, as Type 1 diabetes can occur at any age. Hence the honus is on measurement of fasting insulin & C- peptide levels in blood which helps to differentiate type of DM [5].

Gestational Diabetes Mellitus (GDM): GDM resembles T2DM in several aspects. GDM occurs in 2- 5% of all pregnancies and may improve or disappear after delivery. About 20- 50% of affected women develop T2DM later in their life [6].

Complications of Diabetes Mellitus: Complications of diabetes are observed in uncontrolled diabetes mellitus [7]. Complications are classified into (1) Acute complications and (2) Chronic complications

1. Acute complications: includes Diabetic Ketoacidosis, hyperglycemia hyperosmolar state, Hypoglycemia, Diabetic coma, Respiratory infections, periodontal disease, etc.

2. Chronic complications: leads to multi-organ damage. Chronic complications of DM are further divided into vascular and non-vascular complications. Vascular complications of DM are subdivided into:

(a) Micro-vascular complications (b) Macro-vascular complications (a) Micro-vascular complications include: Retinopathy, Neuropathy, and Nephropathy

(b) Macro-vascular complications include: Coronary Artery Disease, Peripheral Vascular Disease, Cerebro Vascular Disease etc [8, 9].

Oxidants and Antioxidants

Oxidative stress plays an important role in the development of vascular complications in type 2 diabetes.

Oxidative stress (or oxidant derived tissue injury) occurs when production of oxidants or reactive oxygen species (ROS) exceeds local antioxidant capacity, which includes oxidation of major macromolecules such as proteins, lipids, carbohydrates and DNA. Reactive Nitrogen species (RNS); by- products of nitric oxide (NO) plays a key role in maintaining various physiological functions but can also contribute to several pathological processes at high levels. RNS are comprised of nitrite (NO_2^-), nitrate (NO_3^-) and peroxy nitrite ($ONOO^-$). Studies have suggested that oxidative stress is a common pathogenic factor for the dysfunction of beta and endothelial cells of pancreas [10] Beta cell dysfunction results from prolonged exposure to high glucose, elevated free fatty acid (FFA) levels, or a combination of both. Beta cells are particularly sensitive to ROS as they are low in free-radical quenching (antioxidant) enzymes such as catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD) [11]. Thus, the ability of oxidative stress to damage mitochondria and marked blunt insulin secretion is not surprising.

To prevent ROS/ RNS caused cellular damage, the body has a defense system called antioxidant. Antioxidant systems prevent uncontrolled formation of free radicals and activated oxygen species, or inhibit their reactions with biological structures [12]. Antioxidants are any

substances which can inhibit the oxidation of oxidative substrates. Antioxidants include molecules play a role in antioxidant defence, including endogenous (initially synthesized) and exogenous (consumed) antioxidants. They can be divided into two types depending on mechanism of action as chain breaking antioxidants or preventive antioxidants. Preventive antioxidants reduce the rate of chain initiation by deactivating metals, quenching singlet oxygen and removing hydroperoxides, including transferrin, ferritin, ethylenediamine tetraacetic acid (EDTA), ceruloplasmin, catalase, SOD and GPx. Chain breaking antioxidants are the molecules that have the ability to receive or donate an electron from a radical with the formation of stable byproducts including α-tocopherol, ascorbic acid, uric acid and β- carotene [12].

Oxidative Stress-Induced Alterations in Diabetes

Oxidative stress in diabetes mellitus causes several adverse effects on the cellular physiology. This is particularly relevant and dangerous for the islet, which is among those tissues that have the lowest levels of intrinsic antioxidant defences. Multiple biochemical pathways and mechanisms of action have been implicated in the deleterious effects of chronic hyperglycemia and oxidative stress on the function of vascular, retinal, and renal[]. tissues [13-14] Here we have described the oxidative stress-induced alterations in major biomolecules in the cell and status of plasma antioxidant potential during type 2 diabetes (Figure 1).

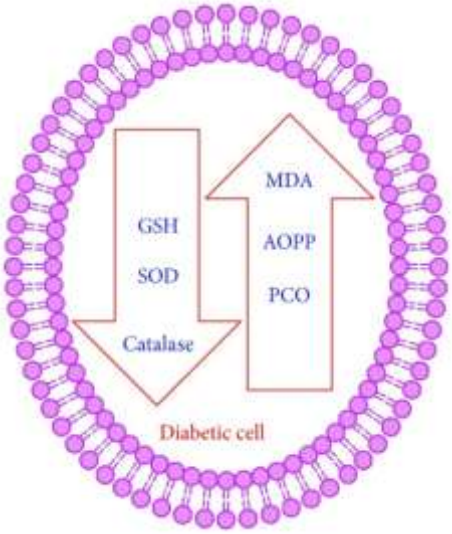

FIGURE 1; Schematic representation of the status of oxidative stress markers during diabetes. MDA: malondialdehyde, AOPP: Advanced oxidation protein products, PCO: protein carbonyls, GSH: reduced glutathione, and SOD: superoxide dismutase.

Lipid Peroxidation

Lipids are reported as one of the primary targets of ROS. Hydroperoxides have toxic effects on cells both directly and through degradation to highly toxic hydroxyl radicals. They may also react with transition metals like iron or copper to form stable aldehydes, such as malondialdehyde (MDA), that damage cell membranes [15]. Peroxidation of lipids produces highly reactive aldehydes, including MDA, acrolein, 4-hydroxynonenal (HNE), 4-oxononenal (ONE), and isolevuglandins (IsoLGs) [16]. It has been reported that peroxyl radicals can remove hydrogen from lipids, producing hydroperoxides that further propagate the free-radical pathway[17].

Protein Oxidation

Proteins are the important vital biomolecules of the cell. They are involved in many physiological functions including cell signalling and transport across the cells. Proteins are another potential target of ROS, whose structure and function can be affected by modification. There are many side chain targets for protein oxidation including cysteine, methionine, and tyrosine. Carbonyls are the oxidation product of proteins and are reported as the potent biomarker of oxidative stress [18]. They represent the stable end product generated upon formation of transient radical species, such as chloramines and nitrogen/carbon radicals, which are induced by oxidant stimuli. Glycation has been reported to induce the formation of protein carbonyls, such as ketoamine derivatives, thus generating reactive radicals and perpetuating a vicious cycle [19].

Glutathione Level

Glutathione (GSH), a tripeptide, γ-L-glutamyl-L-cysteinylglycine, is present in all mammalian tissues at 1–10 mM concentrations (highest concentration in liver) as the most abundant nonprotein thiol that defends against oxidative stress [20]. GSH can maintain SH groups of proteins in a reduced state, participate in amino acid transport, detoxify foreign radicals, act as coenzyme in several enzymatic reactions, and also prevent tissue damage [21].

Catalase

Catalase is an antioxidative enzyme present nearly in all living organisms. It plays an important role against oxidative stress-generated complications such as diabetes and cardiovascular diseases [22].

Superoxide Dismutase

Superoxide dismutase (SOD) is the antioxidant enzyme that catalyses the dismutation of superoxide anion (O_2^-) into hydrogen peroxide and molecular oxygen [23]. All mammalian tissues contain three forms of SOD: Cu-Zn-SOD, Mn-SOD, and extracellular EC-SOD, and each of them is a product of a distinct gene [24].

The present study will be done to oxidative stress and its correlation with glycated hemoglobin in patients of type 2 diabetes mellitus.

REVIEW OF LITERATURE

Diabetes mellitus is a metabolic disorder characterized by hyperglycemia along with biochemical alterations of glucose and lipid peroxidation. It produces free radicals that induce lipid peroxidation which acts as an indicator for oxidative stress in the body. The widely used assay for lipid peroxidation involves measurement of malondialdehyde (MDA). Defensive system in the body consists of antioxidant enzymes which help in scavenging free radicals. Two such antioxidant enzymes are reduced glutathione (GSH) and superoxide dismutase (SOD) [25].

This study was Conducted on Oxidative stress in patients with diabetes mellitus and found that the Oxidative stress in a biological system is the shift in the homeostasis between oxidants and antioxidants in favour of oxidants. It is suggested to be playing a key role in onset and development of complication of Diabetes Mellitus. The present study aimed to assess the oxidative stress and compare the antioxidant enzyme status in patients with Type 2 Diabetes and paired controls. Total study population consisted of 33 subjects with Diabetes and 10 controls from both male and female gender incorporated for the study. Plasma Malondialdehyde level, an indicator of oxidative stress and antioxidative enzyme system including Erythrocyte Superoxide dismutase, catalase and Glutathione Peroxidase were estimated. Subjects with diabetes had significantly higher plasma malondialdehyde as compared to controls. Antioxidant enzymes levels were found to be lower in diabetic patients as compared to controls. In conclusion, hyperglycemia increases production of free radicals leading to increased lipid peroxidation. The antioxidant enzyme system weakens in diabetic patients compared to controls. It was noted that patients with good glycemic control had less oxidative stress compared to patients with poor control[26]

The study was Conducted on Lipid Peroxidation and Lipid Profile in Type II Diabetes Mellitus designed to find out the relation between lipid peroxidation, lipoprotein levels to severity and complication of diabetes mellitus. Degree of lipid peroxidation was measured in terms of malondialdehyde (MDA) along with antioxidants, lipid profile and blood glucose in diabetes mellitus. Total 100 human subjects, out of which 50 healthy individuals of age group (50-70years) were taken as control & 50 elderly diabetic subjects of age group (50-70years) were taken as cases. There was significant increase in the lipid profile except HDL cholesterol, which is decreased, Also significant decrease in antioxidant enzymes such as Reduced glutathione,

glutathione peroxidase, glutathione reductase, superoxide dismutase except catalase was seen as compared to the control subjects. Other findings observed was that the level of lipid peroxide (MDA) increased as per the increase in concentration of blood glucose. Our findings indicate that the increase in the lipid peroxidation product MDA and decline in glutathione-dependent antioxidant defences may appear early in non insulin dependent type 2diabetes mellitus patients.[27]

This study was conducted on Impact of hyperglycaemia on molecular markers of oxidative stress and antioxidants in type 2 diabetes mellitus and found that the pathogenesis of type 2 diabetes mellitus (T2DM) is strongly linked to oxidative stress mainly caused by chronic hyperglycaemia. The present study investigates the association between hyperglycaemia with oxidative stress markers, antioxidants and lipid profile. The case-control study involved two groups, T2DM patients (n = 83) and age and sex matched controls (n = 81). Serum levels of various molecular markers malondialdehyde (MDA), reactive oxygen species (ROS) and nitric oxide (NO), superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), vitamin C, total antioxidant capacity (TAC) and lipid parameters total cholesterol, triglycerides, low density lipoprotein (LDL) and high density lipoprotein (HDL) were measured using spectrophotometric assays. Results were analysed to compare and correlate glycaemic levels with these molecular markers. Results. T2DM patients had a higher body mass index (BMI) and body fat percentage. 2 hour blood glucose, glycated haemoglobin A1c % (HbA1c), total cholesterol, triglycerides and LDL were higher in diabetics, HDL was found to be lower in diabetics than in controls. Mean levels of enzymatic and non-enzymatic antioxidants SOD, CAT, GSH, vitamin C and TAC were significantly lower while oxidative stress markers NO, ROS and MDA were higher in T2DM patients. NO showed a positive correlation ($r = 0.3993$, $p < 0.0001$) whereas TAC showed a negative correlation with glycaemia ($r = –0.4796$, $p < 0.0001$) [28]

This study was conducted in our study there is a disturbed lipid profile in Type-2 DM patients. Serum total cholesterol, Triglycerides, LDL, level found increased while HDL level decreased. Hence, high levels of TC, TG, LDL and low levels of HDL may be due to insulin resistance and other influencing factors like obesity, increase calorie intake, genetic, sedentary lifestyle, lack of muscular activity, in type-2 DM patients. In our study we concluded that poor glycemic control, dyslipidemia due to insulin resistance contributed to the increased oxidative stress. In our study increased MDA levels indicate the increased oxidative stress. Enzymatic

antioxidant like SOD activity is significantly decreased in our study. Non enzymatic antioxifdant vitamin C and E levels are also decreased. From this we can conclude that decreased erythrocyte SOD activity, decreased vitamin C and E levels indicate the reduced antioxidant status in Type-2 DM patients. Hence dietary supplementation of vitamin C and E may be helpful to improve antioxidant status of the body. [29]

AIM AND OBJECTIVES

1. Correlation of Oxidative Stress with various parameters in diabetic and non diabetics.
2. Status of Lipid Peroxidation in diabetic and non diabetics.
3. Correlation between Oxidation Stress with duration and complications of type2 diabetes mellitus

MATERIAL AND METHODS

STUDY SETTING

The Present Study will be Conducted in Department of Biochemistry of Government Medical College and Guru Nanak Dev Hospital Amritsar.

STUDY POPULATION

The present study will be conducted on individuals of either Sex visiting the Medicine OPD/Ward , Guru Nanak Dev Hospital Amritsar, for various routine biochemical parameters.

STUDY DESIGN

The present study is a descriptive Study.

SAMPLE SIZE

Total 200 (100 diabetic patients and 100 non diabetic individuals)

- The sample size formula (Daniel, 1999) is used, which is

$$n = \frac{Z^2 P(1-P)}{d^2}$$

- If your population more than 10.000

  Where

- Z : statistic for a level of confidence. (for the level of confidence of 95%, which is conventional, z value is 1.96).
- P: expected prevalence or proportion. (P is considered 0.5)
- d: precision. (d is considered 0.05 to produce good precision and smaller error of estimate)

Sample Size Calculation:

A Confident Interval of 95% will give a Z-Score = 1.96

Margin of Error = +/- 4.43%.

Prevalence of type 2 diabetes in India = 0.116

= ((1.96)2 x 0.116(0.884)) / (0.0443)2

= (3.8416 x 0.102) / 0.00196

= 0.394 / 0.00196

= 200.9

200 respondents are needed

INCLUSION CRITERIA

1. Diabetics: Patients with type II diabetics mellitus confirmed by fasting blood sugar, under medication (under hypoglycemic drugs and insulin) in the age group of 30-60 years.
2. Controls: Normal healthy non diabetic individuals in age group of 30-60 years.

EXCLUSION CRITERIA

The subjects with liver disease, renal disease , thyroid disease, tuberculosis, hypertension, pancreatitis, Coronary artery disease (CAD, previous history) Stroke, individuals on drugs like gluco-corticoids, Nicotinic acid, Thyroid hormones, β adrenergic antagonists and thiazide diuretics, drug addicts patient with endocrinopathies such as acromegaly, patients with down syndrome were excluded from the present study.

SAMPLING TECHNIQUE/METHOD

Blood samples will be collected under aseptic condition, 5 ml of venous Fasting blood sample will be collected from each individual into a vial. The blood sample will be centrifuged at 3000rpm for 10minutes to separate serum or plasma. The clear supernatant will be used for the biochemical analysis of plasma Glucose, Serum lipid profile and EDTA whole blood sample used for hbA1c, SOD, Glutathione peroxidase and plasma MDA, Catalase.

METHODOLGY;

Principle of Plasma Glucose (GOD/POD METHOD)[30]

Glucose is oxidized to Gluconic Acid and Hydrogen Peroxide in presence of glucose oxidase. Hydrogen Peroxide further reacts with phenol and 4 –Aminoantipyrine by the catalytic action of peroxidase to form a red coloured quinoneimine dye complex. The intensity of the coloured formed is directly is proportional to the amount of glucose present in the sample.

Glucose + O_2 ----^{GOD}-----> Gluconic Acid + H_2O_2

H_2O_2 + Phenolic. + 4 – Aminoantipyrine ----^{POD}-----> Quinoneimine dye + H_2O

Principle of Blood HbA1c (Ion Exchange Resin Method)[31]

A haemolysed preparation of the whole blood is mixed continuously for 5 minutes with weak binding cation exchange resin. During this time, HbAo binds to the resin. After the mixing period, a filter is used to separate the supernatant containing the glycohemoglobin from the resin. The Glycohaemoglobin percent is determined by measuring the absorbances at 415nm of the glycohaemoglobin fraction and the total haemoglobin fraction. The ratio of the two absorbances gives the percentage Glycohaemoglobin.

Principle of Cholesterol (CHOD/ PAP METHOD) [30]

When the serum is reacted with the polyethylene glycol contained in the precipitating reagent, all the VLDL and LDL are precipitated. The HDL remains in the supernatant and is then assayed as a sample for cholesterol using the cholesterol (CHOD/PAP) reagent.

Principle of Triglycerides (GPO/ PAP METHOD) [30]

Lipoprotein lipase hydrolyses triglycerides to glycerol and free fatty acids. The glycerol formed with ATP in the presence of glycerol kinase forms glycerol 3 phosphate which is oxidized by the enzyme glycerol phosphate oxidase to form hydrogen peroxide. The hydrogen peroxide further reacts with phenolic compound and 4 aminoantipyrine by the catalytic action of peroxidase to form a red coloured quinoneimine dye complex. Intensity of the colour formed is directly proportional to the amount of triglycerides present in the sample.

Principle HDL Cholesterol (PEG Precipitation Method)[30]

When the serum is reacted with the polyethylene glycol contained in the precipitating reagent, all the VLDL and LDL are precipitated. The HDL remains in the supernatant and is then assayed as a sample for cholesterol using the cholesterol (CHOD/PAP) reagent.

Calculation of LDL cholesterol (mg/dl) (freidewalds formula)[30]

$$= (\text{Total Cholesterol} - (\frac{\text{triglycerides}}{5}) - (\text{HDL Cholesterol})$$

Principle of Lipid Peroxide (Method : Thiobarbituric Acid Assay)[32]

In this method the lipid peroxide level was inferred in terms of the level of Malondialdehyde (MDA). Serum or plasma containing MDA is treated with thiobarbituric acid (TBA) in presence of 40% trichloroacetic acid (TCA). After heating in boiling water for 10 mins, the resulting pink coloured MDA-TBA adduct is read colorimetrically at 530 nm.

Principle Superoxide Dismutase[33]

The role of superoxide dismutase (SOD) is to accelerate the dismutation of the toxic superoxide radical (O2), produded during oxidative energy processes, to hydrogen peroxide and molecular oxygen. This method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T.) to form a red formazan dye. The superoxide dismutase activity is then measured by the degree of inhibition of this reaction. One unit of SOD is that which causes a 50% inhibition of the rate of reduction of INT under the conditions of the assay.

Principle of Glutathione Peroxidase; UV Method[34]

This method is based on that of Paglia and Valentine. Glutathione Peroxidase (GPX) catalyses the oxidation of Glutathione (GSH) by Cumene Hydroperoxide. In the presence of Glutathione Reductase (GR) and NADPH the oxidized Glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP* . The decrease in absorbance at 340 nm is measured.

Principle of Catalase [35]

Catalase activity was measured by incubating the enzyme sample in 1.0 mL substrate (20 µmol per ml hydrogen peroxide in 60 mmol/l sodium-potassium phosphate buffer, pH 7.4) at 37o C for three minutes. The reaction was stopped with titanium sulphate. The method quantifies undecomposed hydrogen peroxide by reacting it with a titanium sulphate reagent to produce pertitanic acid. Absorbance of the yellow pertitanic acid was measured at 405 nm against the blank.

Principle of Albumin (BCG Method) [36]

Albumin in a buffered solution reacts with the anionic bromocresol green (BCG) with a dye binding reaction to give a proportionate green colour which is measured at 628 nm (600-650). The final colour is stable for 10 minutes.

Principle of Uric acid (enzymatic method) [37]

Uric acid is converted by uricase into allantoin and hydrogen peroxide which in presence of peroxidase (POD) oxidizes the chromogen to a red coloured compound which is read at 500 nm (492-550). The final colour of the reaction is stable for 15 minutes.

Principle of Ascorbic acid (Dinitrophenylhydrazine Method) [38]

The ascorbic acid is converted to dehydroascorbic acid by shaking with norit and this is then coupled with 2, 4- dinitrophenylhydrazine in presence of thiourea as a mild reducing agent. Sulphuric acid then converts the dinitrophenylhydrazone into a red compound which is assayed colorimetrically.

Principle of Total Bilirubin (jendrassik & grof Method) [39]

Bilirubin reacts with diazotized sulphanilic acid to form an azocompound the colour of which is measured at 546 nm (530-560) and is proportional to the concentration of bilirubin. For Total Bilibrubin the reaction is accelerated by caffeine reagent. The readings for Total & Direct Bilirubin are taken after 5 min. of incubation. The Total Bilirubin reaction is stable for 10 minutes.

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